



**Alternative methods for agribusiness
Analytical performances certified**

**VALIDATION CERTIFICATE FOR ALTERNATIVE ANALYTICAL METHOD
ACCORDING TO STANDARD EN ISO 16140: 2003**

Certificate No.: EUR 15/03 - 12/05

Validation date* :	09.12.2005
Extension date*:	15.12.2006
Renewal date :	04.12.2009
End of validity :	09.12.2013

** EN ISO 16140 protocol was used in 2005 and 2006 for the preliminary study and in 2006 for the interlaboratory study.*

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is hereby authorized to refer to this **AFNOR Validation certificate** for the following alternative qualitative analysis method:

LUMIPROBE 24 Listeria monocytogenes

Protocol reference: FTLMT-V-12/09 (TUBE)
 FTLMP-V-12/09 (MICROPLAQUE)

SCOPE

All human food products (*except French cheeses "cantal" and "salers"*) and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 11290-1 (1997) including **amendment A1** (2004) – Food microbiology - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method.

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PRINCIPLE OF THE METHOD

The *LUMIPROBE 24 Listeria monocytogenes* method is a test which associates an enrichment step in a specific broth with a hybridisation of nucleic probes in solid phase, allowing the rapid and specific detection of the *Listeria monocytogenes*. The RNA of the targeted bacteria, released by lysis, is captured by an oligonucleotide fixed on a coated support. It is then combined by hybridisation with a second oligonucleotide labelled by a tracer. Hybrids are then revealed by a luminescent reaction.

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed by one of the following means:

- According to classical tests described in methods standardized by CEN or ISO (including a purification step), starting from the enrichment broth (RM broth)
- By inoculating the enrichment broth (RM broth) on a chromogenic agar with *Listeria* Agar Ottaviani-Agosti formula, or on a chromogenic agar issued from a method certified according to the AFNOR VALIDATION mark (ALOA[®] and RLM were used during the validation study)

In the event of discordant results (positive with alternative method, non-confirmed by tests described above) the laboratory must follow the necessary steps to ensure the validity of the obtained result.

Note (History of validation)

1/ The validation extension of December 2006 addresses the modification of the enrichment protocol for all non dairy and non raw products and the extension of the scope of the method to all human food products (except French cheeses "Cantal" and "Salers") and environmental samples.

The reported results take into account the results from the preliminary study conducted in 2005. The interlaboratory extension study has been conducted in compliance with the European standard EN ISO 16140.

Two protocols have been tested: one, as the general protocol, and the other, specific for raw products.

2/ During the renewal study of 2009, no supplementary tests were done. Since the last validation, the protocol *LUMIPROBE 24 Listeria monocytogenes* method was not modified. The reference method and the protocol of validation remained unchanged.

Relative ACCURACY, relative SPECIFICITY and relative SENSITIVITY Comparison of performances of the alternative method and of the reference method

In 2005, some tests were carried out on 62 product samples, of which 15 were naturally contaminated, 15 artificially contaminated, and 32 non-contaminated, belonging to the "dairy products" food category.

In 2006, some supplementary tests have been carried out on 242 samples of food products of which 60 were naturally contaminated, 63 artificially contaminated, and 119 non-contaminated, belonging to the following general food categories:

Meat products, vegetables products, sea food products and environmental samples.

All samples were analysed **in single** by the **two methods**.

Table of results (Cf. Table 1 of the EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 135 ⁽¹⁾	Positive deviation A+ / R- PD = 7 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 11 ⁽²⁾	Negative agreement A- / R- NA = 151 ⁽³⁾

(1) Confirmed positives

(2) Of which no sample presumed positive by the alternative method was negative after confirmation

(3) Of which 3 samples presumed positive by the alternative method were negative after confirmation

Percentages obtained compared to the reference method are as follows:

- Relative accuracy: **AC = 94%**
- Relative specificity: **SP = 96%**

NB: A relative specificity below 100% results from a number of confirmed supplementary positives and not from false positives

- Relative sensitivity: **SE = 92%**

Sensitivity was also recalculated taking into account all confirmed positives (including the supplementary positives with the alternative method):

Alternative method:
 $(PA + PD) / (PA + PD + ND) = 93\%$

Reference method:
 $(PA + ND) / (PA + PD + ND) = 95\%$

Analysis of discrepant results (according to annex F of EN ISO 16140):

PD = 7 ; ND = 11 , as a result $Y = PD + ND = 18$
 $6 \leq Y \leq 22$; $m = 5$; $M = 2$, as a result $m > M$

Conclusion

Both methods are not different in statistical term.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Some tests were carried out in 2005, on the combination dairy products/*Listeria monocytogenes* isolated from raw milk. This product is representing the "dairy product" category.

Supplementary tests were carried out in 2006, on the four combinations food products/strains as described in the following table. These products represent the following food product categories: meat products, vegetables products, sea food products and environmental samples.

Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination.

Results obtained are as follows:

Matrix	Strain	Relative detection level LOD_{50} (3) With confidence interval (CFU/25g or 25 ml)	
		Alternative method	Reference method
Dairy products	<i>Listeria monocytogenes</i>	1.9 [1.0 – 3.6]	0.5 [0.4 – 0.8]
Smoked salmon	<i>Listeria monocytogenes</i> 3a	2.2 [1.4 – 3.5]	1.3 [0.8 – 2.1]
Rillettes	<i>Listeria monocytogenes</i> 1/2a	0.9 [0.6 – 1.5]	0.6 [0.4 – 0.9]
Mixed raw vegetables	<i>Listeria monocytogenes</i> 1/2c	0.6 [0.4 – 2.0]	0.5 [0.3 – 0.8]
Rinsing water	<i>Listeria monocytogenes</i> 1/2a	0.7 [0.6 – 0.9]	0.6 [0.4 – 0.9]

(3) LOD_{50} : estimation of level of contamination enabling positive detection by alternative method in 50% of cases.

"Hitchins A. Proposed Use of a 50% Limit of detection Value in Defining Uncertainty Limits in the Validation of Presence-Absence Microbial detection Methods, Draft 10th December, 2003"

Conclusion:

The detection level of the alternative method is set between 0.6 and 2.2 UFC/25g.
The detection level of the reference method is set between 0.5 and 1.3 UFC/25g.

INCLUSIVITY / EXCLUSIVITY**Implementation of alternative method only**

- In 2006, 50 strains of *Listeria monocytogenes* were detected out of 50 tested.
- In 2005 the study of 34 strains not belonging to the genus *Listeria monocytogenes* did not detect the presence of any cross-reactions.

PRACTICABILITY**Implementation of alternative method only**

- **Response time :**
 - **Positive** results using the alternative method are obtained in 2 days (*in case of a positive result obtained by Lumiprobe 24, confirmed in 24h using a chromogenic agar*) or 9 days (*if confirmed with classical tests of the reference method, with purification step included*), against 4 to 12 days using the reference method.
 - **Negative** results are obtained in 1 day using the alternative method against 2 to 5 days using the reference method.
 - In the case of results presumed positive using the alternative method, but rendered negative following confirmation, these negative results are obtained in 2 to 5 days.

INTER-LABORATORY STUDY (in compliance with EN ISO 16140 Standard)

The inter-laboratory study was conducted in 2006 with 11 participating laboratories. The analysis were carried out on samples of pork patés, artificially contaminated with a strain of *Listeria monocytogenes* 1/2b at the following 3 levels of contamination:

- Level 0 CFU/25g
- Level 3 CFU/25g
- Level 30 CFU/25g

The laboratories tested, using **both methods**. **8 replicate samples** for each level of contamination, giving a total of 48 analysis for each participating laboratories.

The following results were obtained:

Contami- nation level	Total number of samples	Number of samples analysed	Number of results exploited *	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	88	88	80	80	80	0	0
1	88	88	80	0	1**	80	79**
2	88	88	80	0	0	80	80

* One laboratory has been excluded because some negative samples have been found positive with the reference method and the hypothesis of a cross contamination has been verified.

** One laboratory has obtained a result below the threshold value for a sample corresponding to the lowest rate of contamination.

Calculations

- Relative accuracy = **99%**
- Specificity = **100%**
- Sensitivity = **99%**

Interpretation

Results of the inter-laboratory study are comparable to those obtained during the preliminary study.

Sensitivity was also recalculated taking into account all confirmed positive results (this includes supplementary positives with alternative method):

Alternative method :	Reference method :
$(PA + PD) / (PA + PD + ND) = 99\%$	$(PA + ND) / (PA + PD + ND) = 100\%$

Accordance, concordance and concordance odds ratio:

Accordance: percentage chance of finding the same result (i.e. both negative or both positive) from two identical test portions analysed in the same laboratory, under repeatability conditions (i.e. one operator using the same apparatus and same reagents within the shortest feasible time interval). The accordance is the average (mean) of the probabilities that two replicates give the same result for each laboratory.

Concordance: percentage chance of finding the same result for two identical samples analysed in two different laboratories. The concordance is the percentage of all pairings of duplicates giving the same result.

Concordance odds ratio (COR): defined by the following formula:
 $COR = \text{accordance} \times (100 - \text{concordance}) / \text{concordance} \times (100 - \text{accordance})$

The following table indicates values for the **alternative method**:

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1.00
L1	98%	97%	1.52
L2	100%	100%	1.00

The following table indicates values for the **reference method**:

Contamination level	Accordance	Concordance	COR
L0	100%	100%	1
L1	100%	100%	1
L2	100%	100%	1

Conclusion

Variability of the alternative method (accordance, concordance, concordance odds ratio) is equivalent to that of the reference method.

Please send any queries concerning the performance of the validated method to
AFNOR Certification.

You may download a summary document on the preliminary and inter-laboratory
studies on www.afnor-validation.com